



Comprehensive chemotaxonomic analysis of saffron crocus tepal and stamen samples, as raw materials with potential antidepressant activity

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ABSTRACT

Saffron crocus (*Crocus sativus* L.) has been widely grown in Iran. Its stigma is considered as the most valuable spice for which several pharmacological activities have been reported in preclinical and clinical studies, the antidepressant effect being the most thoroughly studied and confirmed. This plant part contains several characteristic secondary metabolites, including the carotenoids crocetin and crocin, and the monoterpenoid glucoside picrocrocin, and safranal. Since only the stigma is utilized industrially, huge amount of saffron crocus by-product remains unused. Recently, the number of papers dealing with the chemical and pharmacological analysis of saffron is increasing; however, there are no systematic studies on the chemical variability of the major by-products.

In the present study, we harvested saffron crocus flowers from 40 different locations of Iran. The tepals and stamens were separated and subjected to qualitative and quantitative analysis by HPLC-DAD. The presence and amount of seven marker compounds, including crocin, crocetin, picrocrocin, safranal, kaempferol-3-O-sophoroside, kaempferol-3-O-glucoside, and quercetin-3-O-sophoroside were determined.

The analytical method was validated for filter compatibility, stability, suitability, accuracy, precision, intermediate precision, and repeatability. Tepal and stamen samples contained three flavonol glycosides. The main constituent of the tepals was kaempferol-3-O-sophoroside (62.19–99.48 mg/g). In the stamen, the amount of flavonoids was lower than in the tepal. The amount of kaempferol-3-O-glucoside, as the most abundant compound, ranged between 1.72–7.44 mg/g. Crocin, crocetin, picrocrocin, and safranal were not detected in any of the analysed samples.

Our results point out that saffron crocus by-products, particularly tepals might be considered as rich sources of flavonol glycosides. The data presented here can be useful in setting quality standards for plant parts of *C. sativus* that are currently considered as by-products of saffron production.

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1. Introduction

Saffron crocus (*Crocus sativus* L., Iridaceae) is cultivated in some Asian and European countries, in highest extent in Iran. The stigma of this plant (known as saffron) is a popular spice and has also been applied in the traditional Arabic and Islamic medicine for several purposes, especially as cardiac and liver tonic and hep-

atic deobstruent, to facilitate difficult labour and for the treatment of female genito-urinary disorders and male impotence. Its effects on the central nervous system have also been recorded; however, old texts have to be reinterpreted according to the current conceptions of medicine and pharmacology. According to Sayyed Esma'il Jorjani (1042–1136 A.D.) saffron is astringent and resolvent and its fragrance can strengthen these two effects. Hence, its action on enlivening the essence of the spirit and inducing happiness is great. This supposed activity might be translated as a positive effect on mood or as an antidepressant effect [1].

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There is an overlap between the traditional uses and the evidence-based applications of saffron. The clinical efficacy of saffron has been studied in diabetes [2], age-related macular degeneration [3,4], cognitive impairment [5–8], glaucoma [9], sexual dysfunction in women [10] and men [11], and premenstrual syndrome [12]. However, most of the studies assessed its antidepressant activity and the efficacy in mild to moderate depression has been confirmed by a recent meta-analysis as well [13].

Crocin, crocetin, picrocrocin and safranal are the main characteristic compounds of saffron stigma. The colour of saffron is due to the carotenoids crocin and crocetin, the specific bitterish taste is attributed to the monoterpene glycoside picrocrocin, whereas safranal, an aromatic aldehyde of the volatile oil contributes to the aroma [1].

Saffron is known as the most expensive spice in the world, 300 000 flowers are approximately required to obtain 1 kg of dried stigma [14]. Considering its price and the increasing scientific interest for the bioactivities of saffron, the analysis of alternative plant parts (the industrial by-products tepal and stamen), that are available in larger amounts and hence are cheaper, seems to be promising approach. Several papers have reported the constituents of major saffron crocus by-products. It has to be noted, that several papers report the chemical composition of sepal and petal samples, however, botanically these plant parts should be defined as tepal. Therefore, in case of previous papers we refer to the plant parts used by the authors. The petals of the plant were characterized by a high total phenolic and flavonoid content compared to stamens and styles [14,15]. Both sepals and stamens of samples from different regions of Italy were characterized with kaempferol-3-*O*-sophoroside as main flavonoid constituent [16]. From the flower material of saffron crocus (except stigma) kaempferol-3-*O*-sophoroside was isolated as the major component [17]. Beside flavonols, anthocyanins are also present in the floral bioresidues of saffron crocus as major constituents, delphinidin 3,5-di-*O*-glucoside being the predominant compound [18]. From a methanolic extract of tepals, kaempferol glycosides comprising kaempferol 3-*O*-sophoroside, kaempferol 3-glucoside, and kaempferol 3-*O*- β -D-(2-*O*- β -D-6-acetylglucosyl)glucopyranoside-7-*O*- β -D-glucopyranoside were isolated as the major compounds [19]. Kaempferol 3-*O*-sophoroside was identified as main component by utilizing HR-MAS NMR spectroscopy as well [20]. Other studies have also reported flavonoids from tepals. Kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-glucoside, and quercetin-3-*O*-sophoroside were reported in these studies [21,22].

The antidepressant activities of tepals have been studied in animal experiments. Both the aqueous and ethanolic extracts of stigma and tepal decreased immobility time in comparison with normal saline in the forced swimming test in mice [23]. Kaempferol, a flavonoid of the tepals was reported to have antidepressant activity on mice and rats in the same test [24].

The efficacy of tepals has been confirmed in two clinical trials. In a randomized, double-blind trial, 40 patients with mild to moderate depression were treated either with 30 mg saffron crocus tepal or 20 mg fluoxetine for 8 weeks. The herbal preparation was similarly effective as fluoxetine with remission rates of 25 % in both groups [25]. In a similar trial with 40 patients, the efficacy of the tepals (30 mg) was compared to placebo. After 6 weeks of treatment, the tepal was more effective than placebo in improving the severity of depression using the Hamilton Depression Rating Scale [26].

Considering the increasing scientific and industrial interest for saffron crocus by-products, the aim of our study was to systematically analyse the composition of tepal and stamen samples. We developed and validated an HPLC/DAD method for the analysis of seven marker compounds previously identified as the major constituents of saffron crocus stigma, tepal and stamen. Crocin, crocetin, picrocrocin, safranal and

flavonoids [kaempferol-3-*O*-sophoroside (K.S.), kaempferol-3-*O*-glucoside (K.G.), quercetin-3-*O*-sophoroside (Q.S.)] were assessed qualitatively and quantitatively in 40 tepal and stamen samples collected from different regions of Iran.

2. Materials and methods

2.1. Plant materials

Saffron crocus (*Crocus sativus* L.) tepal and stamen samples were collected from 40 different locations of Iran at the same harvesting period in November 2018. All were dried under shade, then the tepals were accurately separated from stamens. The plant samples were individually packed in the sealed plastic bags and stored at room temperature. The growth locations, altitudes, and coordinates of the harvested plant materials are shown in Table 1. For comparison, a commercial saffron stigma sample was also analyzed (Bahraman Co., Mashhad, Iran).

2.2. Chemicals and reagents

All solvents were of analytical grade. Kaempferol-3-*O*-glucoside (K.G.) (Santa Cruz Biotech, California, USA), quercetin-3-*O*-sophoroside (Q.S.), crocetin (trans-crocetin, 98 %), and picrocrocin (Carbosynth, Berkshire, UK), safranal, and crocin (crocetin digentio-biose ester, Sigma-Aldrich, St. Louis, Missouri, USA) were purchased in analytical grade. Kaempferol-3-*O*-sophoroside (K.S.) was isolated in our laboratory, its structure and purity was determined by NMR.

2.3. Extract preparation

20 mg of tepal, 10 mg of stigma, and 50 mg of stamen samples were extracted with the solvent mixture EtOH-H₂O 1:1, using an ultrasonic bath for 15 min, then diluted with the above solvents to 10.0 mL (tepal) and 5.0 mL (stigma and stamen) in volumetric flasks, respectively.

In case of filtered samples, the extracts were filtered via a filter membrane (PTFE-L syringe filter, hydrophilic, FilterBio®, diameter: 13 mm, pore size: 0.45 μ m), the first 1 mL was unused, and the rest 1.5 mL was analysed by HPLC-DAD. In case of centrifuged samples, centrifugation was performed by using DLAB D1008 instrument (7000 rpm) for 1 min. For all samples, three extracts were prepared and analysed in triplicate.

2.4. HPLC apparatus and measurement conditions

HPLC-DAD analysis was carried out on a Shimadzu SPD-M20A HPLC (Shimadzu Corporation, Kyoto, Japan), equipped with a Shimadzu SPD-M20A photodiode array detector, an on-line degasser unit (Shimadzu DGU-20A5R), a column oven (Shimadzu CTO-20AC column oven) and autosampler (Shimadzu SIL-20A8T) using a RP Kinetex® C8 column (5 μ m, 100 Å, 150 \times 4.6 mm, Phenomenex, Torrance, USA) at 30 °C. Chromatographic elution of the samples was accomplished with a gradient solvent system by changing the ratio of MeOH in H₂O (containing 0.066 % of H₃PO₄) as follows: 30 % (0–1 min), 30–57 % (1–7 min), 57–76 % (7–12 min), 76–100 % (12–13 min), keeping at 100 % for 1 min, 100 to 30 % (14–15 min) and keeping at 30 % for 3 min at a flow rate of 1.5 mL/min. The samples were monitored in the UV-VIS range (190–800 nm) and at the UV_{max} of the standards (picrocrocin: 247 nm, Q.S.: 360 nm, K.S.: 354 nm, K.G.: 348 nm, crocin: 441 nm, safranal: 316 nm, and crocetin: 427 nm). Data assessment and acquisition were performed with the LabSolutions (Version 5.82) software (Shimadzu, Kyoto,

Table 1
Provenances of saffron samples.

Code	Location	Altitude (m)	Latitude	Longitude
1T, 1S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
2T, 2S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
3T, 3S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
4T, 4S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
5T, 5S	Dehouye, Meshkan, Neyriz, Fars	1567	29° 15' N	53° 56' E
6T, 6S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
7T, 7S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
8T, 8S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
9T, 9S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
10T, 10S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
11T, 11S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
12T, 12S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
13T, 13S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
14T, 14S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
15T, 15S	Meshkan, Neyriz, Fars	2167	29° 28' N	54° 20' E
16T, 16S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
17T, 17S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
18T, 18S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
19T, 19S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
20T, 20S	Dehchah, Neyriz, Fars	2188	29° 13' N	54° 26' E
21T, 21S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
22T, 22S	Abadeh Tashk, Neyriz, Fars	1608	29° 48' N	53° 43' E
23T, 23S	Neyriz, Fars	1606	29° 11' N	54° 19' E
24T, 24S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
25T, 25S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
26T, 26S	MahmoudAbad, Neyriz, Fars	1579	29° 14' N	54° 17' E
27T, 27S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
28T, 28S	Roniz, Estahban, Fars	1593	29° 11' N	53° 46' E
29T, 29S	HasanAbad, Neyriz, Fars	1579	29° 44' N	53° 54' E
30T, 30S	Estahban, Fars	1797	29° 7' N	54° 2' E
31T, 31S	Estahban, Fars	1797	29° 7' N	54° 2' E
32T, 32S	Estahban, Fars	1797	29° 7' N	54° 2' E
33T, 33S	Estahban, Fars	1797	29° 7' N	54° 2' E
34T, 34S	Estahban, Fars	1797	29° 7' N	54° 2' E
35T, 35S	Estahban, Fars	1797	29° 7' N	54° 2' E
36T, 36S	Estahban, Fars	1797	29° 7' N	54° 2' E
37T, 37S	Ghaen, Khorasan e Jonoubi	1445	33° 43' N	59° 11' E
38T, 38S	Torbatejam, Khorasan e Razavi	894	35° 14' N	60° 37' E
39T, 39S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E
40T, 40S	Dehdez, Khoozestan	1439	31° 42' N	50° 17' E

T: tepal; S: stamen.

Japan). 10 and 20 μ L of tepal and stamen extracts were injected, respectively.

2.5. System validation

Validation of our analytical method was carried out according to the ICH Harmonised Guideline [27] and completed with further experiments. Validation was performed by establishing the calibration curves of seven reference compounds, determining the limit of detection and quantification values, assessing system suitability, accuracy, precision, repeatability, stability and filter compatibility of the extracts or pure compounds.

3. Results and discussion

3.1. Validation

Our goal was to develop and validate an analytical method that is suitable for the analysis of saffron stigma samples and saffron crocus by-products. Marker compounds that were used as reference standards during our experiments were chosen based on literature data as follows: crocin, crocetin, picrocrocin, safranal, K.S., K.G., and Q.S. During validation, tepal samples and the mixture of the reference compounds were used, and where it was possible, validation was carried out for all the analytes. However, since saffron crocus tepal did not contain crocin, crocetin, picrocrocin, and safranal, validation was partial for these compounds.

3.1.1. Calibration curve and linearity

Seven major components of saffron crocus, K.S., K.G., Q.S., crocetin, picrocrocin, safranal, and crocin were used to establish calibration curves and limit of detection (LoD) and limit of quantitation (LoQ) values (Table 2). Calibration curves are based on 8–11 calibration points. The correlation coefficient of the calibration curves was at least 0.998. Calibration curves covered 2 orders of magnitude of analyte concentration.

3.1.2. Filter compatibility

To select the best method for sample preparation, one tepal specimen was extracted by ultrasonic bath, then filtered or centrifuged. Except for crocetin, sample preparation by filtration or centrifugation had no major impact on quantitative results; however, in case of this compound, the amount of the analyte decreased with 17.3 % as the result of filtration. For the other analytes, slight higher values were measured in filtered samples (picrocrocin: 100.86 ± 0.35 %; Q.S.: 101.14 ± 0.31 %; K.S.: 100.89 ± 0.29 %; K.G.: 100.20 ± 1.4 %; crocin: 101.21 ± 0.38 %; safranal: 100.27 ± 1.37 %).

3.1.3. Stability

To evaluate the stability of the solutions of reference compounds, the standard mixtures were prepared by filtration or centrifugation, stored at 4 °C and room temperature (23 °C), then injected at day 0, 1, 3, 5, and 7 (Table 3). In case of picrocrocin and the flavonoids, storage time and temperature did not affect the concentration of the analytes, whereas in case of crocin and

Table 2

Calibration curve characteristics and limit of detection and quantification values.

Standard	LoD ($\mu\text{g}/\text{inj}$)	LoQ ($\mu\text{g}/\text{inj}$)	Calibration points	Range covered ($\mu\text{g}/\text{inj}$)	Regression equations	R ²
Picrocrocin	0.00245	0.00741	8	0.03–0.9	$y = (1.10320 \times 10^9)x - 10690.0$	0.9997394
Q.S.	0.02554	0.07739	10	0.082–6.15	$y = (6.55841 \times 10^8)x - 5275.02$	0.9996089
K.S.	0.02277	0.06901	9	0.076–3.04	$y = (5.96019 \times 10^8)x - 20062.4$	0.9996702
K.G.	0.06763	0.20495	9	0.1075–4.3	$y = (1.36039 \times 10^8)x - 4682.70$	0.9984738
Crocin	0.00128	0.00386	10	0.0145–0.58	$y = (2.92163 \times 10^9)x - 13570.0$	0.9994265
Safranal	0.00177	0.00536	8	0.08–2.4	$y = (2.06147 \times 10^9)x + 37991.1$	0.9982921
Crocetin	0.00032	0.00097	11	0.00172–0.129	$y = (9.35271 \times 10^9)x - 5778.24$	0.9999475

Table 3

Stability of the dissolved reference compounds after 1, 3, 5 and 7 days (values compared to 100 % day 0 values).

Sample	Days	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
23 °C	1	100.97	101.08	100.92	101.77	101.18	99.84	86.28
	3	104.06	100.59	100.69	101.80	95.93	87.89	76.71
	5	100.37	99.97	100.53	102.74	91.29	85.89	76.47
	7	100.59	99.70	101.12	105.00	90.65	83.71	78.94
4 °C	1	100.51	100.66	100.56	101.05	100.84	100.29	86.52
	3	104.09	100.66	100.56	101.82	100.57	99.74	85.92
	5	100.69	100.41	100.67	102.30	100.30	96.44	84.48
	7	100.77	100.89	101.27	104.98	100.50	94.64	85.44

Table 4

Results of the system suitability tests.

	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Safranal	Crocetin
RSD% of AUC	0.47	0.18	0.43	2.53	0.38	0.41	1.11
Tailing factor	1.139–1.145	1.043–1.067	1.113–1.127	1.356–1.408	1.177–1.910	1.171–1.184	1.310–1.334
RSD% of RT	0.66	0.62	0.36	0.22	0.19	0.14	0.10

RT: retention time.

Table 5

Recovery values (%) of the controls.

Level	Picrocrocin (stigma)		Q.S.		K.S.		K.G.		Crocin (stigma)		Safranal (stigma)		Crocetin (stigma)	
	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%	%	RSD%
50 %	111.92	0.27	106.54	0.12	100.95	0.29	96.09	0.62	–	–	–	–	99.54	1.33
100 %	101.80	0.07	112.25	0.10	99.60	0.06	103.36	2.03	97.89	0.27	–	–	83.69	1.73
150 %	110.32	1.32	116.26	0.30	101.54	0.12	97.57	2.77	108.10	0.06	–	–	89.40	0.42

safranal, decomposition was observed especially at room temperature. Interestingly, the concentration of crocetin decreased remarkably after one day, and the temperature had no major influence on this process.

3.1.4. System suitability

The mixture of the reference standards was injected 5 times to assess suitability of the analytical system. The low RSD% values of the AUCs and retention times, together with the tailing factors below confirm that the system is suitable for the measurement of these compounds (Table 4).

3.1.5. Accuracy

The accuracy of the method was assessed by the determination of recoveries (Table 5). Recoveries of the marker compounds was assessed by adding known amounts of the standards to a tepal (or in case of crocin, picrocrocin, crocetin, and safranal: stigma) sample at three different concentrations (50, 100, and 150 % of the previously determined amounts). Three independent samples were prepared for each concentration levels and injected in triplicates. The recovery values ranged between 96.09–111.92 %, 83.69–112.25 %, and 89.40–116.26 % in case of 50 %, 100 %, and 150 % amounts of added analytes, respectively.

3.1.6. Precision

In order to determine the precision of the analytical method, one tepal and one stigma sample was extracted individually and injected 10 times. Precision was determined by calculating RSD% values of the AUCs (Table 6). The RSD% values below 1% confirm the precision of the method.

3.1.7. Repeatability

The repeatability of the experiment was performed by analysis of six tepal sample extracts within 24 h (intra-assay precision). Repeatability was characterized by RSD% values of the whole datasets for Q.S., K.S., and K.G., RSD% values were 3.38 %, 3.71 % and 3.44 %, respectively.

3.1.8. Intermediate precision

The same tepal sample was analysed by two analysts and intermediate precision was determined as RSD% of the means. For Q.S., K.S., and K.G. the RSD% values were 3.81 %, 2.29 %, and 6.85 %, respectively.

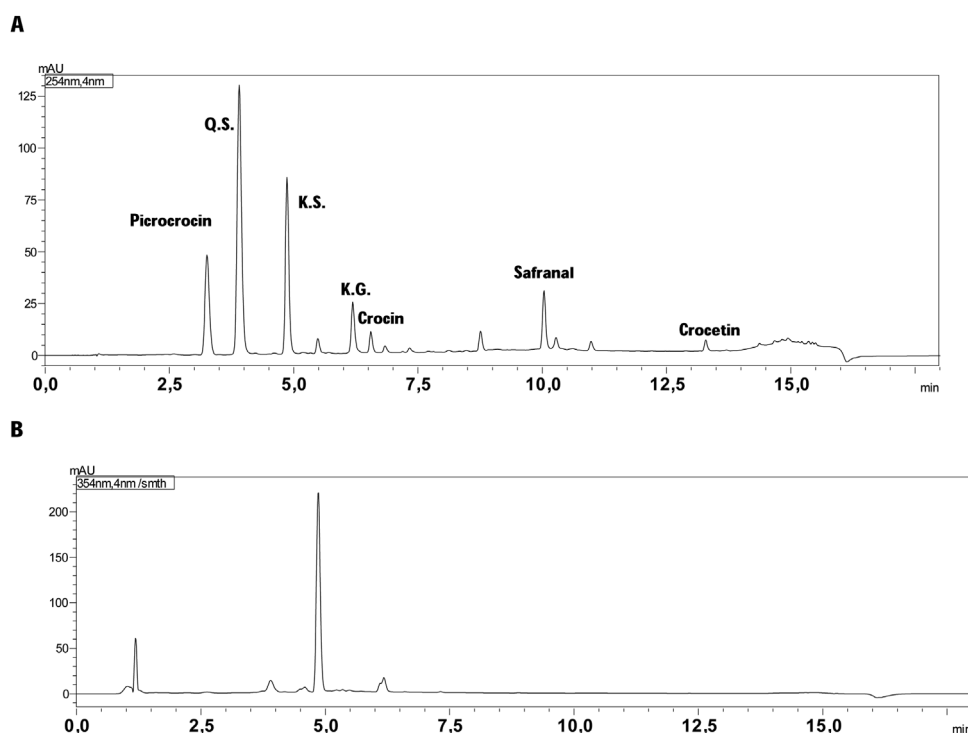
3.2. HPLC/DAD analysis of tepal and stamen samples

HPLC/DAD analysis of 40 different tepal and stamen samples was carried out to qualitatively and quantitatively analyse their selected marker compounds. Our newly developed HPLC method

Table 6

Precision of the analytical method as determined by RSD%.

Samples	Picrocrocin	Q.S.	K.S.	K.G.	Crocin	Crocetin
Tepal	–	1.72	0.07	0.53	–	–
Stigma	1.86	0.30	2.26	2.68	0.58	1.86

**Fig. 1.** HPLC chromatogram of the mixture of reference compounds (A), and of a tepal sample (B) (354 nm).

allowed good separation and hence reliable analysis of these compounds in saffron crocus samples (Fig. 1).

In stamen and tepal samples, three flavonol glycosides, namely Q.S., K.S., and K.G. were detected as major components. For comparison, we analysed a saffron stigma sample as well. From this sample picrocrocin, crocin, and crocetin were identified, which is in line with the previous data. There is only one report on the presence of crocin in saffron crocus tepal; however, very low amount (0.6 %) was reported in the hydrolysed extracts compared to kaempferol (12.6 %) [28]. A comparative study of the stamen and stigma revealed that crocin, picrocrocin, and safranal are not present in stamen, whilst they are major components of the stigma [29].

In Table 7, the amounts of marker compounds in tepal and stamen samples are presented as means (mg/g plant material) together with the standard deviation values. The tepal samples contained Q.S., K.S. and K.G., in the ranges of 6.20–10.82, 62.19–99.48, and 27.38–45.17 mg/g, respectively, whereas the amount of these compounds was lower in the stamen (1.72–6.07, 0.89–6.62 and 1.72–7.44 mg/g, respectively). K.S., as the major constituent of saffron crocus tepal was present in the highest amount in sample 1 with an amount of 99.48 mg/g. Tepal sample 23 contained the highest amount of Q.S. (10.82 mg/g), while tepal sample 5 contained the lowest amounts of Q.S. and K.S. with 6.21 and 62.19 mg/g, respectively. The content of K.G. in tepal samples was remarkably different from 27.74 to 45.18 mg/g analysed in samples 40 and 1, respectively. In general, the stamen samples contained less flavonoids than the tepals. The highest amount of Q.S. in stamens was observed in sample 29 with 6.08 mg/g, whilst sample 3 contained the lowest concentration (1.63 mg/g). The content of K.S.

ranged from 6.62 mg/g (sample 40) to 0.93 mg/g (sample 3) in the stamen. The quantity of K.G. was also variable, the highest and lowest amounts were determined in sample 31 and 8 with 7.44 and 1.72 mg/g, respectively.

Our results confirmed some former findings. K.S., K.G., and Q.S. were described as major or characteristic flavonoid components of flowers and tepals by several authors [16,17,19–20,21,22,29–31]. Flavonol derivatives (6–10 mg/g) were characterized as the major compounds of stamen and tepal samples harvested from two different region of Italy. K.S. was the major compound (6.41–8.30 mg/g of fresh tepals and 0.37–1.70 mg/g of fresh stamen) [16].

The comparison of our results to those published previously has some limitations. *Crocus* flowers, having monocot characteristic structure, consist of stamens, stigmas and tepal. However, numerous studies refer to perianth as “sepal” and “petal.” In some cases the investigated flower part is not clearly named, however, the description narrows it to tepals [21,22,32]. Some of the studies are referring to the investigated sample as “petals” without any further description [19,20,25,26], while some further studies refer to saffron flower without stigma [17,28,30,31].

3.3. Classification of saffron crocus populations

Cluster analysis (CA) and principal component analysis (PCA) were performed to characterize and classify saffron crocus tepal and stamen samples harvested from different locations in Iran according to their Q.S., K.S., and K.G. contents. According to the CA analysis, the saffron crocus samples collected from various locations were classified in three major groups demonstrating three distinct chemotypes based on their flavonoid contents. Chemotype

Table 7
Levels of marker compounds in saffron samples.

Sample	Q.S.		K.S.		K.G.	
	T	S	T	S	T	S
1	10.24 ± 0.23	4.23 ± 0.14	99.48 ± 0.07	1.82 ± 0.1	45.18 ± 0.24	3.65 ± 0.21
2	6.29 ± 0.04	3.49 ± 0.14	76.40 ± 1.37	1.47 ± 0.06	37.31 ± 0.77	2.47 ± 0.12
3	6.23 ± 0.15	1.63 ± 0.18	62.41 ± 1.41	0.93 ± 0.02	33.71 ± 0.98	2.42 ± 0.46
4	9.19 ± 0.32	5.60 ± 0.2	74.82 ± 3.32	1.53 ± 0.26	36.01 ± 1.6	3.69 ± 0.15
5	6.21 ± 0.2	4.56 ± 0.2	62.19 ± 2.06	2.21 ± 0.09	35.73 ± 1.23	4.88 ± 0.29
6	7.92 ± 0.34	2.11 ± 0.12	71.81 ± 3.67	1.03 ± 0.03	39.25 ± 2.27	2.05 ± 0.01
7	7.61 ± 0.30	1.72 ± 0.02	77.35 ± 3.96	1.03 ± 0.06	37.38 ± 1.73	2.01 ± 0.11
8	9.43 ± 0.23	1.98 ± 0.06	75.00 ± 0.55	0.90 ± 0.03	37.30 ± 0.46	1.72 ± 0.12
9	8.68 ± 0.29	1.80 ± 0.06	74.88 ± 2.84	1.20 ± 0.03	34.09 ± 1.09	2.72 ± 0.24
10	8.26 ± 0.10	1.79 ± 0.04	71.45 ± 1.51	1.03 ± 0.0	32.08 ± 0.07	2.17 ± 0.09
11	8.51 ± 0.38	2.02 ± 0.08	68.55 ± 1.88	1.20 ± 0.04	32.08 ± 0.82	2.33 ± 0.05
12	10.26 ± 0.03	2.15 ± 0.1	82.91 ± 2.41	1.07 ± 0.07	38.75 ± 0.44	2.76 ± 0.09
13	8.24 ± 0.04	4.43 ± 0.15	72.94 ± 2.02	2.49 ± 0.13	32.58 ± 0.61	4.00 ± 0.08
14	8.37 ± 0.34	5.16 ± 0.06	79.83 ± 0.13	1.78 ± 0.04	35.34 ± 0.56	4.30 ± 0.06
15	7.10 ± 0.08	4.65 ± 0.2	77.79 ± 0.33	2.24 ± 0.25	38.77 ± 0.47	3.72 ± 0.22
16	9.14 ± 0.48	4.45 ± 0.11	86.50 ± 0.85	1.64 ± 0.06	34.83 ± 0.06	3.45 ± 0.09
17	8.34 ± 0.41	4.77 ± 0.11	72.03 ± 3.65	1.68 ± 0.06	30.28 ± 1.62	4.39 ± 0.23
18	7.47 ± 0.28	5.95 ± 0.6	72.28 ± 3.47	1.51 ± 0.01	34.49 ± 1.82	3.55 ± 0.1
19	7.56 ± 0.07	3.54 ± 0.11	83.01 ± 0.74	4.63 ± 0.26	38.17 ± 0.3	5.94 ± 0.01
20	7.91 ± 0.02	5.32 ± 0.14	86.22 ± 2.77	3.08 ± 0.12	37.44 ± 1.21	5.00 ± 0.14
21	9.25 ± 0.17	5.08 ± 0.19	85.59 ± 2.04	3.40 ± 0.02	37.59 ± 0.82	4.16 ± 0.14
22	7.45 ± 0.21	4.10 ± 0.23	79.01 ± 2.93	2.09 ± 0.13	33.23 ± 1.12	4.67 ± 0.22
23	10.82 ± 0.38	4.53 ± 0.19	98.99 ± 2.85	5.21 ± 0.22	36.44 ± 0.88	7.31 ± 0.05
24	7.24 ± 0.04	4.82 ± 0.03	67.80 ± 2.72	2.07 ± 0.0	37.28 ± 0.19	5.34 ± 0.53
25	8.25 ± 0.22	4.55 ± 0.24	79.30 ± 1.54	1.99 ± 0.09	40.96 ± 1.4	3.11 ± 0.09
26	7.58 ± 0.17	5.52 ± 0.13	71.07 ± 0.07	2.04 ± 0.07	33.31 ± 0.43	5.19 ± 0.1
27	8.63 ± 0.07	4.43 ± 0.08	75.66 ± 2.47	2.06 ± 0.06	33.22 ± 1.11	5.15 ± 0.24
28	7.83 ± 0.3	3.95 ± 0.22	76.59 ± 1.52	2.02 ± 0.03	34.77 ± 0.66	4.03 ± 0.17
29	7.44 ± 0.2	6.08 ± 0.36	75.61 ± 1.07	2.20 ± 0.05	36.73 ± 0.73	5.23 ± 0.41
30	7.39 ± 0.23	5.28 ± 0.28	80.46 ± 4.01	2.01 ± 0.02	34.76 ± 1.79	5.96 ± 0.43
31	7.16 ± 0.06	4.29 ± 0.21	87.52 ± 1.5	3.09 ± 0.19	42.51 ± 1.15	7.44 ± 0.31
32	8.37 ± 0.26	5.14 ± 0.3	74.16 ± 1.03	2.33 ± 0.13	33.61 ± 1.3	4.83 ± 0.01
33	7.83 ± 0.06	4.43 ± 0.08	75.77 ± 1.56	2.24 ± 0.13	36.99 ± 0.63	4.71 ± 0.15
34	8.35 ± 0.1	4.49 ± 0.3	74.16 ± 1.12	2.45 ± 0.17	34.69 ± 0.42	4.80 ± 0.25
35	7.83 ± 0.33	5.74 ± 0.09	75.86 ± 0.83	2.19 ± 0.08	41.03 ± 0.47	5.13 ± 0.05
36	9.43 ± 0.36	4.77 ± 0.13	88.63 ± 3.41	3.34 ± 0.19	43.68 ± 1.13	5.10 ± 0.11
37	6.75 ± 0.04	5.40 ± 0.31	76.00 ± 1.31	1.83 ± 0.1	34.32 ± 0.39	5.70 ± 0.05
38	8.96 ± 0.12	5.13 ± 0.15	81.03 ± 3.35	1.96 ± 0.1	36.87 ± 1.0	6.78 ± 0.04
39	6.87 ± 0.11	2.87 ± 0.06	74.44 ± 1.2	2.59 ± 0.15	29.04 ± 1.19	4.30 ± 0.22
40	6.42 ± 0.21	2.86 ± 0.12	73.91 ± 2.85	6.62 ± 2.06	27.74 ± 1.22	5.26 ± 1.03

The amounts are presented as mg of samples; Q.S.: quercetin-3-O-sophoroside; K.S.: kaempferol-3-O-sophoroside; K.G.: kaempferol-3-O-glucoside; T: tepal; S: stamen.

I was characterized with the highest Q.S. content of tepal samples, including the populations 2, 3, 6–12, and 39, samples belonging to chemotype II contained high quantity of K.G. in tepal samples (populations 4, 5, 13, 14, 16–18, 22, 24, 26–30, 32–34, and 37), whereas chemotype III was the richest in Q.S., K.S., and K.G. in the stamens, and K.S. in the tepals (populations 1, 15, 16, 19–21, 23, 25, 31, 35, 36, 38, 40). The results of the PCA are presented on a dendrogram (Fig. 2).

Eigenvalues, variances and cumulative variances of the four PCs are listed in Table 8. In accordance with PCA analysis, four principal components (PC): PC1, PC2, PC3, and PC4 explained 89.17 % of total variation. As demonstrated in Table 8, PC1 described 25.17 % of total variation which had positive correlation with SQS (0.96 %) and SKG (0.75 %). Interestingly, a positive correlation between PC2 with SKS (0.92 %), SKG (0.57 %) and PKS (0.52 %) was recorded by 24.22 % of variance. Furthermore, PC3 and PC4 explained 22.09 % and 17.69 % of total variance, respectively, while the most positive correlations in PC3 and PC4 were observed with PQS (0.80 %) and PKG (0.96 %), respectively.

Biosynthesis of secondary metabolites may be influenced by different environmental factors (e.g. UV irradiation, temperature) at different altitudes. The impact of altitude on the composition of certain plant species has previously been studied. For example, we have confirmed that the apigenin and luteolin content of *Matri-*

Table 8
Eigenvalues, variance and cumulative variance for four principal components.

Flavonoids	Principal components			
	PC1	PC2	PC3	PC4
PQS	−0.02	−0.06	0.80	0.13
PKS	0.13	0.52	0.78	0.32
PKG	0.07	0.05	0.25	0.96
SQS	0.96	0.02	0.06	0.09
SKS	0.12	0.92	0.07	0.48
SKG	0.75	0.57	−0.05	−0.01
Eigenvalue	1.51	1.45	1.33	1.06
Variance (%)	25.17	24.22	22.09	17.69
Cumulative variance (%)	25.17	49.39	71.49	89.17

PQS: Quercetin-3-O-sophoroside in tepal; PKS: Kaempferol-3-O-sophoroside in tepal; PKG: Kaempferol-3-O-glucoside in tepal; SQS: Quercetin-3-O-sophoroside in stamen; SKS: Kaempferol-3-O-sophoroside in stamen; SKG: Kaempferol-3-O-glucoside in stamen.

caria chamomilla L. was significantly higher at higher altitude [33]. The diversity of total phenolic and flavonoid contents of different Iranian *Ferulago angulata* (Schltdl.) Boiss. populations also confirm the influence of altitude on the chemodiversity of plants [34]. The volatile oil composition may also be affected by altitude [33–36]. In case of saffron crocus, this is the first report on chemodiversity of samples from different geographical locations.

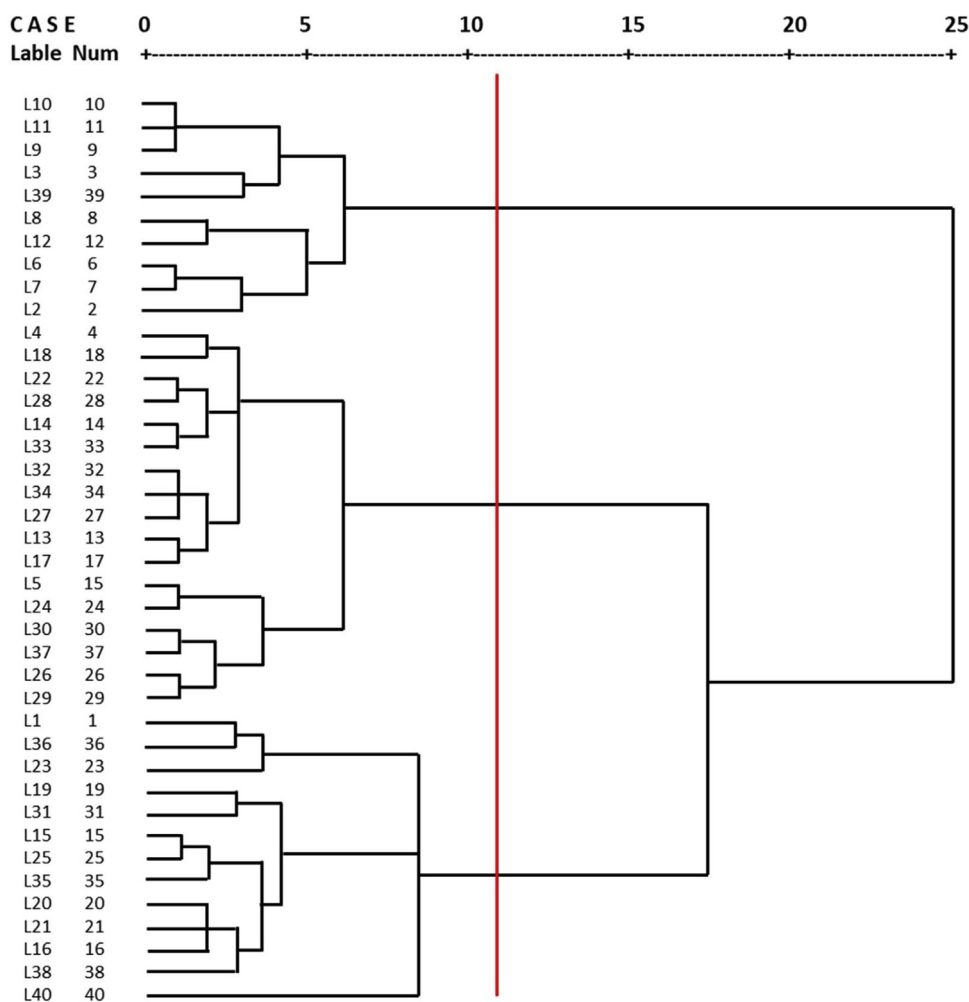


Fig. 2. Dendrogram of cluster analysis (based on Euclidean distances) of the saffron samples: chemotype I (Q.S in tepal), chemotype II (K.G in tepal), chemotype III (Q.S in stamen, K.S in stamen, K.G in stamen, K.S in tepal).

4. Conclusion

The industrial and scientific interests for saffron crocus by-products, including tepals and stamens have considerably been increased, due to the high price of saffron stigma and the new data on bioactivities of tepals. Here we report the development and validation of an HPLC-DAD method that allows the assessment of saffron crocus samples based on the analysis of seven marker compounds (K.S., K.G., Q.S., picrocrocin, crocin, crocetin, and safranal). A recent study reports the metabolomic fingerprinting of saffron by using LC-MS, which can be the basis of the authentication of this spice [37]. LC-MS was used also for the analysis of saffron crocus by-products, ie. tepals [31], tepals, stamens and flowers [32], leaves, tepals, spaths, corm, and tunics [38], however, this is the first report on the analysis of a series of samples of different geographic origin. The flavonol glycosides K.S., K.G., and Q.S. can be utilized as qualitative and quantitative marker compounds, their total amount in dry tepal and stamen samples ranged between 62.19–99.48 mg/g and 0.90–6.62 mg/g for K.S., 27.74–45.18 mg/g and 1.72–7.44 mg/g for K.G., and 6.21–10.82 mg/g and 1.63–6.08 mg/g for Q.S., respectively. These ranges were established based on the analysis of 40 different Iranian tepal and stamen samples. K.S. was the main component of tepals (62.19–99.48 mg/g), while K.G. (1.72–7.44 mg/g) was the predominant constituent of the stamen samples. Characteristic compounds of the stigma (picrocrocin, crocin, crocetin,

and safranal) could not be detected in tepal and stamen samples; however, our method allows their determination as well.

The biplot was prepared based on the two PCs by 89.17 % of variance. As it was shown in Fig. 3, saffron crocus samples were grouped in three classes that approved the CA analysis. According to the PCA and CA analyses, some of the samples from long distances, for example samples 40 and 38 deriving from locations with different climate, were classified in the same group (III), while others from the same location, Meshkan (population 12 in class I, Meshkan samples 13 and 14 in group II, Meshkan 15 in group III), were classified in separate groups. Saffron crocus is propagated by vegetative method which may affect plant diversity. It seems that the analyzed flavonoids were not affected by genotype and climate condition, however, edaphic factors, water and nutrition management might be responsible for the chemodiversity of the studied samples.

CRedit authorship contribution statement

Javad Mottaghipisheh: Data curation, Formal analysis, Investigation, Writing - original draft. **Mohammad Mahmoodi Sourestani:** Software, Investigation, Visualization, Writing - original draft. **Tivadar Kiss:** Data curation, Investigation, Project administration. **Attila Horváth:** Formal analysis, Investigation. **Barbara Tóth:** Data curation, Validation. **Mehdi Ayanmanesh:**

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